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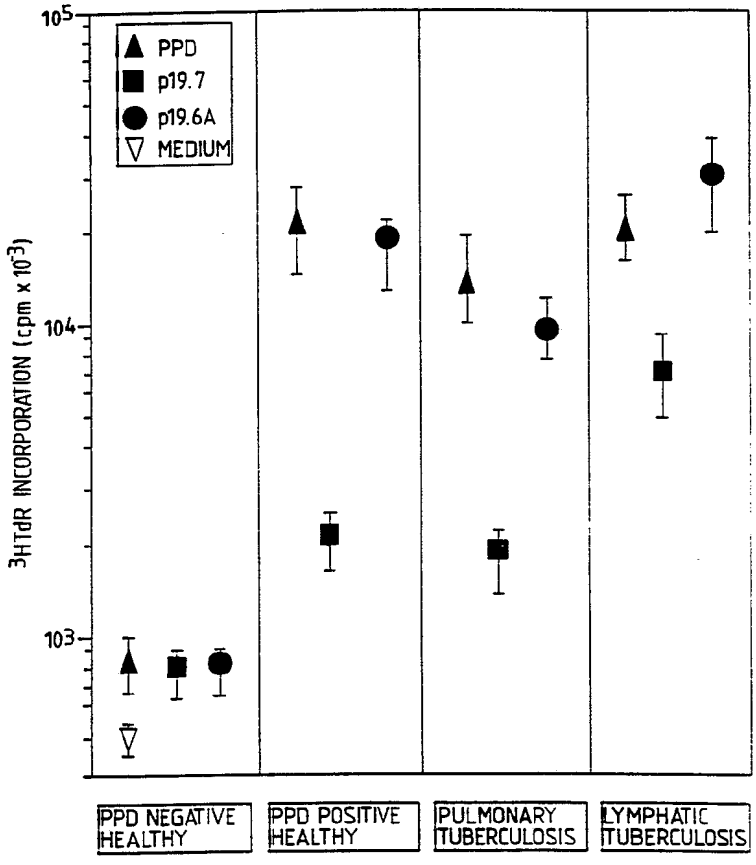
(54) Title: DIAGNOSTIC PEPTIDES DERIVED FROM M.TUBERCULOSIS ANTIGENS

(57) Abstract

The present invention relates to peptides derived from the 38kDa and 19kDa tubercule bacilli protein of M.tuberculosis. One such peptide, 38.G of the sequence (I): Asp-Gln-Val-His-Phe-Gln-Pro-Leu-Pro-Pro-Ala-Val-Val-Lys-Leu-Ser-Asp-Ala-Leu-Ile has been found to be useful for the diagnosis of tuberculosis and provides a basis for a method of distinguishing TB patients from infected or otherwise sensitised but healthy clinical suspects.



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DIAGNOSTIC PEPTIDES DERIVED FROM M.TUBERCULOSIS ANTIGENS

The present invention relates to certain peptides and their use in diagnosis of tuberculosis.

Tuberculosis is a mycobacterial disease of humans and animals. In humans the pathogen Mycobacterium tuberculosis is responsible for widespread morbidity and mortality in the developing countries and is still prevalent despite vaccination programmes in the developed world. Early diagnosis is important to ensure that appropriate treatment is applied. The present diagnostic test, the purified protein derivative (PPD) "tuberculin" skin test, is highly sensitive (i.e. detects nearly all positives) but is not sufficiently specific in that it also identifies healthy individuals who are sensitised to antigens from M. tuberculosis (due to environmental exposure to M. tuberculosis or other cross-reacting mycobacteria, vaccination or recovery from previous infections) and therefore fails to distinguish them from tuberculosis patients (i.e. it identifies many false positives).

In animals the disease causes considerable economic losses in farm livestock such as cattle, deer and donkeys and is generally controlled by slaughtering animals suspected to be infected or believed to carry the disease (for instance wild animals such as badgers) to other animals and humans. Again the current diagnostic test gives many false positives, resulting in unnecessary slaughtering.

There is therefore a need to replace the PPD test. However, the ease and sensitivity of these tests are much appreciated by medical and veterinary workers and any replacement must not compromise these aspects if it is to gain acceptance.

The PPD tuberculin-based skin tests rely upon the use of soluble antigens obtained from different strains of tubercle bacilli as test reagents. Investigations into mycobacterial antigens have revealed a plethora of potentially immunogenic materials but so far no suitable antigen has been identified which could satisfactorily replace PPD in diagnosis of M. tuberculosis infections.

The present inventors have identified a number of peptide sequences within the known 38kDa lipoprotein antigen of M.

tuberculosis which can be used in place of PPD and which provides improved specificity in diagnosing M.tuberculosis infections. It is believed that these peptides will also provide improved specificity for diagnosis of tuberculosis in other species.

Accordingly, in a first aspect, the present invention provides a peptide comprising the sequence (I)

Asp-Gln-Val-His-Phe-Gln-Pro-Leu-Pro-Pro-
-Ala-Val-Val-Lys-Leu-Ser-Asp-Ala-Leu-Ile (I)

or a variant or immunological equivalent of the sequence (I), or a fragment thereof which includes the sequence (I'):

His-Phe-Gln-Pro-Leu-Pro-Pro-Ala-Val-Val, or a variant or immunological equivalent thereof. Unless otherwise specified below, reference hereafter to the peptide comprising the sequence (I) also includes reference to peptides comprising the sequence (I').

The sequence (I) corresponds to residues 350 to 369 of the 38kDa lipoprotein antigen of M.tuberculosis, and the sequence (I') corresponds to residues 353 to 362, see Fig. 1.

Variants of the sequence (I) have at least one deletion, insertion or substitution with respect to the sequence (I), preferably not more than five deletions, insertions or substitutions, for instance two, three or four deletions, insertions or substitutions. Deletion or insertion of two residues, even if contiguous, counts as two deletions or insertions and so on.

Immunological equivalents of the sequence (I) have the same function and utility as sequence (I) but may be completely unrelated in amino acid sequence. Techniques are now available for synthesis of random peptide sequences and for screening such sequences for particular biological activities. It is therefore within the ability of those skilled in the art to obtain a monoclonal antibody against the sequence (I) and to use this to screen a series of random peptides, starting from the sequence (I), a variant thereof or a completely unrelated sequence and thus to generate and select peptides which will be recognised by such an antibody. Peptides so recognised will be candidates for use as immunological equivalents according to the present

invention. However, as the PPD test (and any replacement therefor) rely upon T-cell mediated reactions, such candidate peptides will require further screening and selection based upon a T-cell recognition protocol, for instance the lymph node proliferation assay described below.

In a further aspect, the invention provides a peptide comprising the sequence (II):

Phe-Asn-Leu-Trp-Gly-Pro-Ala-Phe-His-Glu-
Arg-Tyr-Pro-Asn-Val-Thr-Ile-Thr-Ala (II)

or a variant or immunological equivalent (as defined in relation to the peptide of sequence (I)) thereof.

The sequence (II) corresponds to residues 65 to 83 of the 38kDa lipoprotein of Figure 1, and is also referred to below as the peptide I.

In a yet further aspect, the invention provides a peptide comprising the sequence (III):

Asp-Ala-Ala-Thr-Ala-Gln-Thr-Leu-Gln-Ala-Phe-
Leu-His-Trp-Ala-Ile-Thr-Asp-Gly-Asn (III)

or a variant or immunological equivalent (as defined in relation to the peptide of sequence (I)) thereof.

The sequence (III) corresponds to residues 325-342 of the 38kDa protein of Figure 1, and is also referred to below as the peptide K.

In a yet further aspect, the invention provides a peptide comprising the sequence (IV):

Met-Lys-Ile-Arg-Leu-His-Thr-Leu-Leu-Ala-Val-
Leu-Trp-Ala-Ala-Pro-Leu-Leu-Leu-Ala (IV)

or a variant or immunological equivalent (as defined in relation to the peptide of sequence (I)) thereof.

The sequence (IV) corresponds to residues 1 to 20 of the 38kDa protein of Figure 1, and is also referred to below as the peptide A.

The peptides of the present invention comprise a sequence

(I) or a fragment thereof containing the sequence (I'), (II), (III) or (IV), a variant or immunological equivalent thereof. The peptides may comprise further sequences at either or both the N- and C-terminals of the sequence (I), (I') (II), (III) or (IV) or variant or immunological equivalent thereof. Such further sequences may be related to the 38kDa protein or may be carrier proteins, marker sequences or other peptide sequences unrelated to the 38kDa protein sequence provided that such further sequences do not deleteriously affect the function and utility of the peptide.

However the present invention does not extend to known polypeptides such as the 38kDa lipoprotein and the corresponding recombinant polypeptide lacking the lipid moiety. Nevertheless, peptides containing variants or immunological equivalents of the sequence of formula (I) and which are known entities are excluded only in relation to claims to those entities per se or to the production thereof.

Peptides according to the present invention may be obtained by digestion of the 38kDa lipoprotein of M.tuberculosis and recovery and purification of appropriate fractions, by de novo chemical synthesis and by recombinant DNA techniques, all of which are well known in the field of biotechnology.

The peptides of the present invention are useful in diagnosing tuberculosis by stimulation of lymphocytes which have been sensitised to antigens from a tuberculosis-causing pathogen. Thus the peptides of the invention may be used in in vivo skin-tests relying upon a delayed-type hypersensitivity (DTH) reaction causing an observable reddening and swelling of the skin. Alternatively the peptides of the invention may be used in ex vivo tests based on detection of lymphocyte activation. In both cases the peptides of the invention are advantageous in that they are well defined materials which are highly pure and reproducible in contrast to PPD-tuberculin.

It is also possible that the peptides of the invention may be used in dimerized or polymerized form, either in the form of homodimers or homopolymers, or in the form of heterodimers or heteropolymers. Such forms of the peptides can be made by techniques known in the art, eg. by those methods mentioned above.

It has also surprisingly been found that the peptide of formula (I) fails to provoke a strong immunogenic response in DHT or lymphocyte activation tests in patients with pulmonary and non-lymphatic extrapulmonary tuberculosis. This finding - that the peptide of sequence (I) shows anergy - provides a basis for a method of distinguishing TB patients from infected or otherwise sensitised but healthy clinical suspects.

Accordingly the peptides of the present invention may be used in place of PPD in diagnosing human tuberculosis. When used in this manner, the peptides afford excellent sensitivity and improved specificity.

Sensitivity is defined as the percentage of actual positives which are correctly identified by the diagnostic test. Both PPD and the present peptides afford sensitivities approaching the ideal of 100%.

Specificity is defined as 100 minus the percentage of false positives in the subjects who are actually negative. As previously mentioned, PPD falsely identifies individuals as being positive when they have T-cell immunity as a result of environmental exposure or recovery from tuberculosis. The present peptides provide surprisingly improved specificity since they lead to far fewer false positives.

In lymphocyte activation tests lymphocytes are obtained from the patient in a sample of body fluid such as peripheral blood or lymph or a sample of tissue such as lymph nodes or in a sample from oral, bronchioalveolar or nasal lavage, and exposed to the peptide. Lymphocyte activation may be detected by a variety of techniques known in themselves, such as by proliferation assays as described below or by detection of increased levels of lymphokines produced by the lymphocytes.

In one method of diagnosis, a peptide of the sequence (I) may be used in combination with a peptide of sequence (II) and/or (III) and or (IV) and/or a further peptide from the 38kDa lipoprotein antigen of M. tuberculosis. The result of a lymphocyte activation test using the combination of peptides will be indicative of the status of a patient. For example a weak response to both the peptide of sequence (I) and to a second peptide will generally indicate a PPD negative, healthy patient. However, if the peptide of sequence (I) does not generate a

strong response, while the second peptide does, then this will be an anergic response indicative of pulmonary or non-lymphatic extrapulmonary tuberculosis.

The peptides of the present invention are preferably used as pharmaceutically or veterinarily acceptable compositions comprising a solution or suspension of the peptide in a suitable solvent. The solvent is preferably water for injection for use in humans or distilled water, deionised water or pyrogen-free water for use in animals. The compositions may additionally comprise conventional excipients such as buffers; agents to stabilise the peptides, adjust the tonicity of the solution and/or to enhance the stability or solubility of the peptides such as other proteins, salts and surface active agents; antibiotic and antimicrobial agents; antioxidants; markers and dyes. For use as a solution or suspension the peptides may be presented as concentrates or dry powders (optionally containing such excipients) for dilution, dissolution or suspension in a suitable solvent. In a preferred embodiment the peptides and optional excipients are presented as lyophilised powders. The compositions may be presented in single dose units or, more usually, in multi-dose units.

In a particular aspect of the invention the peptides are presented in the form of a diagnostic kit comprising a peptide-containing composition and optionally containing diluents for the peptide composition; control reagents such as PPD, other peptide antigens or compositions lacking any active component; conventional components and ingredients; instructions for performing the test and the like.

Whilst the peptides of the present invention provide good sensitivity and improved specificity, they do not necessarily enable the diagnostic test to distinguish between patients immunised with BCG (Bacille Calmette Guerin), which is the conventional agent used in vaccinating against human tuberculosis, and patients suffering from tuberculosis. Accordingly the peptides may be used in conjunction with other tests intended to distinguish patients vaccinated with BCG from those not so vaccinated.

The present invention, in further aspects, therefore provides

(a) a pharmaceutical or veterinary composition comprising a solution or suspension, in a suitable solvent, of a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a variant or immunological equivalent thereof as hereinbefore defined;

(b) peptides comprising a sequence of formula (I), (I') (II), (III) or (IV), or a variant or immunological equivalent thereof as hereinbefore defined or compositions thereof for use in a method of diagnosis practised on the human or animal body;

(c) the use of peptides comprising a sequence of formula (I), (I') (II), (III) or (IV),or a variant or immunological equivalent thereof as hereinbefore defined or compositions thereof in the preparation of a medicament for use in a method of diagnosis practised on the human or animal body;

(d) process for producing compositions by admixing a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a variant or immunological equivalent thereof, with a suitable solvent, diluent or carrier therefor;

(e) methods of diagnosing tuberculosis in a human or non-human animal suspected of suffering from tuberculosis, said method comprising

either intradermal injection of an effective, non-toxic amount of a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a variant or immunological equivalent thereof as hereinbefore defined or a composition thereof, to said human or non-human animal;

or contacting lymphocytes from the human or non-human animal with a lymphocyte-activating amount of a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a variant or immunological equivalent thereof as hereinbefore defined, or a composition thereof;

(f) diagnostic kits comprising a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a

variant or immunological equivalent thereof, or a composition thereof, and

(g) processes for producing a peptide comprising a sequence of formula (I), (I'), (II), (III) or (IV), a variant or immunological equivalent thereof.

In a further aspect, the invention also relates to peptides derived from another protein component of tubercule bacilli. This component is the 19kDa protein. We have found that the region from amino acid residues 45 to 80 contains at least two regions of interest. Thus, the present invention provides a peptide designated 19.6A:

Gly-Ala-Ala-Ser-Gly-Pro-Lys-Val-Val-Ile-Asp-Gly-Lys-Asp-Gln-Asn-Val-Thr-Gly-Ser (19.6A)

or a variant or immunological equivalent thereof (as defined above). This corresponds to the residues 45 to 64 of the 19kDa protein. Preferably, the variant contains at least the fragment from residues 54 to 61, which we have identified as being of particular importance.

This peptide was found to produce a strong DTH reaction in humans and thus can be used in place of PPD in the diagnosis of M. tuberculosis.

The invention also relates to the use of the peptide designated 19.7:

Val-Thr-Gly-Ser-Val-Val-Cys-Thr-Thr-Ala-Ala-Gly-Asn-Val-Asn-Ile-Ala-Ile-Gly-Gly (19.7)

or a variant or immunological equivalent thereof (as defined above) in a method of diagnosis of tuberculosis. This peptide corresponds to the residues 61 to 80 of the 19kDa protein. Preferably, the variant contains at least the fragment from 71 to 78, which we have identified as being of particular importance.

The 19.7 peptide is disclosed in Faith et al, Immunology, (1991) 74; 1-7, Harris et al, J. Immunology, Vol 147; 2706-2712 1991; and Ashbridge et al, J. Immunology (1991) 147, 2248-2254. However, we have surprisingly found that the peptide is useful for the selective diagnosis of lymphatic tuberculosis, since this peptide provokes a selectively enhanced response in lymphocyte

activation tests in patients with this form of tuberculosis when compared with either patients with pulmonary tuberculosis or sensitised healthy subjects.

Accordingly, the present invention provides a method for the diagnosis of lymphatic tuberculosis which comprises bringing a sample of the T-lymphocytes of a patient into contact with peptide 19.7 or a variant or immunological equivalent thereof, and measuring the amount of T-lymphocyte activation.

The procedure is usually performed in vivo.

The peptides 19.6A and 19.7 may be used as pharmaceutical or veterinary compositions in the manner described above in relation to peptides (I) to (IV).

The present invention, in further aspects, therefore provides

(a) a pharmaceutical or veterinary composition comprising a solution or suspension, in a suitable solvent, of a peptide comprising a sequence of formula 19.6A or 19.7, a variant or immunological equivalent thereof as hereinbefore defined;

(b) peptides comprising a sequence of formula 19.6A or 19.7, or a variant or immunological equivalent thereof as hereinbefore defined or compositions thereof for use in a method of diagnosis practised on the human or animal body;

(c) the use of peptides comprising a sequence of formula 19.6A or 19.7,or a variant or immunological equivalent thereof as hereinbefore defined or compositions thereof in the preparation of a medicament for use in a method of diagnosis practised on the human or animal body;

(d) process for producing compositions by admixing a peptide comprising a sequence of formula 19.6A or 19.7, a variant or immunological equivalent thereof, with a suitable solvent, diluent or carrier therefor;

(e) methods of diagnosing tuberculosis in a human or non-human animal suspected of suffering from tuberculosis, said method comprising

either intradermal injection of an effective, non-toxic amount of a peptide comprising a sequence of formula 19.6A or

19.7, a variant or immunological equivalent thereof as hereinbefore defined or a composition thereof, to said human or non-human animal;

or contacting lymphocytes from the human or non-human animal with a lymphocyte-activating amount of a peptide comprising a sequence of formula 19.6A or 19.7, a variant or immunological equivalent thereof as hereinbefore defined, or a composition thereof;

(f) diagnostic kits comprising a peptide comprising a sequence of formula 19.6A or 19.7, a variant or immunological equivalent thereof, or a composition thereof, and

(g) processes for producing a peptide comprising a sequence of formula 19.6A or 19.7, a variant or immunological equivalent thereof.

The invention is described below with reference to the figures of the accompanying drawings in which:

Fig.1. shows the sequence of a fragment of the 38kDa lipoprotein antigen of M.tuberculosis

Fig.2. shows the results of lymphocyte stimulation by peptide G of the invention in PPD positive and PPD negative healthy subjects.

Fig.3. shows human T cell proliferative responses elicited by peptides or PPD. PBL were cultured in vitro with p19.6A, p19.7 (50μg/ml) or 10U/ml PPD for five days and radioactive incorporation determined. Results are expressed as mean thymidine incorporation of each group with standard errors indicated by the vertical bars. Medium values (cells with Ag) for each subject group were not statistically different and a mean value is shown (open triangle). Number of subjects per group see table 5.

Fig.4. shows localization of epitope cores identified in p19.6A and p19.7. The overlapping 20mer peptides 19.6A and 19.7 are shown in relation to the amino acid sequence (residues 45-80) of the 19kDa protein. The underlined tetrapeptide VTGS indicates the four residues shared by both p19.6A and p19.7. Epitope cores for p19.6A (residues 54-61) and p10.7 (residues 71-78) were identified in B10.BR mice and are shown as shaded boxes below the sequence. The human epitope core for p19.6A was identified in four different individuals all of whom responded to peptides containing the core residues 54-60. Human epitope core for p19.7 was identified in a single individual and corresponds to residues 72-77. The presence of a potential cathepsin D motif sequence is indicated, the broken line shows the probable cleavage site between Val^{53} and Ile^{54}.

The invention will now be illustrated by the following Examples which are not intended to limit the scope of the claims in any way:

Example 1

The following peptide was synthesised, along with others described below, by simultaneous multiple synthesis [Houghten, R.A. et al., Bio Techniques, 4 : 522(1986)]:

Asp-Gln-Val-His-Phe-Gln-Pro-Leu-Pro-Pro-
-Ala-Val-Val-Lys-Leu-Ser-Asp-Ala-Leu-Ile.

The peptide was cleaved from the resin support in a single step using hydrogen fluoride in the presence of 5% anisole as scavenger [Houghten, R.A. et al., Int. J. Peptide Res., 27 : 673(1986)] and excess scavenger was removed with ether. The peptide was extracted with acetic acid (10%) and purified by gel filtration through SEPHADEX G15 (Registered Trade Mark) in aqueous acetic acid (25%) or aqueous acetonitrile (50%). Homogeneity and purity were confirmed by reverse phase HPLC and amino acid analysis.

Comparative Example 1

The following peptides were synthesised and purified as described in Example 1 using either aqueous acetic acid (25%) or aqueous acetonitrile (50%) as eluents in the gel filtration step according to the solubility of the peptide:

Peptide	Sequence
A	MKIRLHTLLAVLTAAPLLLA
B	NGKVLAAMYQGTIKTWDDPQ
C	KQDPEGWGKSPGFGTTVDFP
D	SPGFGTTVDFPAVPGALGEN
E	GNGGMVTGCAETPGCVAYIG

F AAGFASKTPANQAISMIDGP

H CGSKPPSGSPETGAGAGTVA

Peptides A to F and H above and the peptide of Example 1 (hereafter referred to as peptide G) all correspond to regions of the 38kDa lipoprotein of M.tuberculosis [Young, D.L. et al., Infect. Immun., 54 : 177 (1986)].

Fig. 1 shows the sequence of the 38kDa lipoprotein and indicates the positions of peptides A to H by boxes below the sequence. Amphipathic regions are indicated by black bars above the sequence and residues identical to or representing conservative substitutions or the corresponding residues of the E.coli PhoS protein are shaded.

The biological activity of peptides A to H was investigated as follows:

MATERIALS AND METHODS.

Mice.

BALB/c (H-2^d), C57BL/10 (H-2^b), B10.BR (H-2^k), B10.D2 (H-2^d), and B10.A(3R) (H-2^b) mice were obtained from Olac Harlem Ltd, (Shaws Farm, Bicester, Oxon, GB). Mice were age and sex matched for each experiment.

Antigens.

A soluble extract (MTSE) was prepared from M.tuberculosis H37Ra as described by Jackett, P.S. et al., J. Clin. Microb., 26 : 2313 (1988). A heat-killed preparation of M.tuberculosis H37Ra was obtained from Difco (Detroit, Michigan, USA). M. bovis BCG was grown as a suspension culture in Middlebrook's 7H9 culture medium.

Production of the 38 kDa-Glutathione-S-transferase fusion protein.

The 38 kDa-glutathione-S-transferase fusion protein (GT38) was prepared from cell lysates of E.coli where it was overexpressed. A Nru I fragment encoding part of the 38 kDa antigen, minus the first 42 residues from the N terminus and the last 4 residues from the C terminus, thus not including sequences corresponding to peptides A and H (see Fig. 1, the Nru I fragment

lies between the dotted lines) was cloned as a blunt fragment into the Sma I site of pGEX 1N (Pharmacia Ltd, Uppsala, Sweden). The construct was then transformed into E.coli strain MC 1061. Several of the resulting transformants were grown individually, then induced with 0.5 mM isopropyl-B-D-thiogalactopyranoside for 2-3 h. Cell lysates were analysed by SDS-PAGE and clones producing fusion proteins identified. The insoluble GT38 protein was partially purified by low speed centrifugation to pellet the fusion protein, followed by solubilisation in 8M urea and then dialysed against PBS. After determining the protein concentration, the dialysates were aliquoted and stored at -20°C. SDS-PAGE analysis indicated that about 50% of the total protein concentration of this preparation was represented by the fusion protein.

Immunisation procedure and preparation of Lymph node (LN) cells.

Mice were immunised s.c. in each hind footpad with a total of either 20 μg MTSE, 20 μg killed H37Ra, 50 μg GT38 or 80 μg synthetic peptide in incomplete Freund's adjuvant (IFA, Difco Laboratories). Control animals were immunised with PBS/IFA. After 8-10 days, draining popliteal LN cells were removed and pooled. Single cell preparations were prepared from groups of 3 mice [Corradin, G. et al., J. Immunol., 119 : 1048 (1977)]. LN cells from BCG infected mice were harvested 2 months after footpad infection with 10^6 organisms.

Lymph node proliferation assay.

LN cells were cultured in triplicate with the appropriate antigen in 96well microtiter plates at 4 x 10^5 cells/well in RPMI 1640, (Gibco, Paisley, Scotland), supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 x 10^{-5} M 2-mercaptoethanol. Plates were incubated for 3 days at 37°C in 5% CO_2, pulsed with 37kBq of ^{3}H-thymidine (Amersham International, Amersham, GB) and 6 h later harvested onto glass fiber filter paper. ^{3}H-thymidine incoporation was quantitated by liquid scintillation counting. Results are expressed as means of triplicates and standard deviations did not generally exceed 10% of the means.

T cell lines.

T cell lines were established from LN cells obtained from peptide-immune mice. They were maintained and expanded by alternating weekly cycles of stimulation with the appropriate peptide antigen in the presence of 3 x 10^6 irradiated spleen cells (3000 rad)/well in 24 well plates and then rested without antigen [Boom, W.H. et al., Infect Immun., 55 : 2223 (1987)]. After at least 4 cycles the lines were assayed for antigen-specific proliferative responses using 2.5 x 10^4 T cells/well and 3 x 10^5 irradiated spleen cells/well.

Fluorescence-activated cell sorter analysis.

The phenotype of T cell lines was established by FACS analysis using rat IgG_{2b} mAb specific for the T cell markers Thy1.2, L3T4 (CD4) and Lyt2.2 (CD8), and a FITC-labelled mAb against rat IgG_{2b} (all mAb were obtained from Seralab, Crawley Down, Sussex, U.K.).

RESULTS.

Proliferative responses of LN cells from mice immunised with synthetic peptides.

C57BL/10, B10.BR and BALB/c mice were immunised with each of the eight peptides. After eight days, the draining popliteal LN cells were harvested and then challenged in vitro with the homologous peptide used for immunisation. Peptides F and G were found to be immunogenic whereas the other six peptides did not stimulate proliferative responses. Peptide F was a more potent immunogen in C57BL/10 and B10.BR mice, whereas BALB/c LN cells responded more strongly to peptide G. Peptides F and G were also assayed in B10.D2 and B10.A(3R) mice and were immunogenic. Differences in MHC-haplotypes of the congenic B10 strains tested did not have a profound influence on the magnitude of response to peptides F and G. Treatment of primed LN cells with anti-Thy1.2 mAb and complement abolished proliferative responses to these peptides.

Proliferative responses to synthetic peptides following immunisation with the 38 kDa protein.

C57BL/10 mice were immunised in the footpads with a

recombinant fusion protein containing the M. tuberculosis 38kDa protein (GT38). After eight days, LN cells were removed and then challenged with the synthetic peptides A to H. Peptide G induced strong proliferative responses whereas peptide F failed to stimulate LN cells significantly. Peptide C induced proliferative responses following immunisation with the GT38 protein. None of the other peptides induced proliferation.

Following immunisation with killed H37Ra organisms or infection with live M. bovis BCG, both of which contain the complete 38 kDa protein, only peptides C and G induced proliferative responses of LN cells in vitro. This response pattern corresponds to that obtained following immunisation with the recombinant GT38 antigen and indicates that the amino-terminus of the 38 kDa protein is not immunogenic in mice.

Peptide F and G specific T cell lines.

Long-term T cell lines were established from LN cells of mice which were immunised and subsequently stimulated in vitro with peptides F or G. The cells were maintained in culture by alternating cycles of stimulation with peptide followed by a resting phase without peptide. After at least four cycles, the proliferative responses to various antigens were determined and the cellular phenotype of these T cell lines analysed by FACS analysis. The proliferative responses of T cell lines derived from BALB/c and B10.BR mice were very similar, consequently only the responses of the BALB/c lines are described in detail. The results of FACS analysis of the 38.G specific BALB/c T cell line demonstrated its phenotype as Thy1.2$^+$, CD4$^+$, CD8$^-$. All other T cell lines were found to be of the same phenotype.

The cell lines derived from peptide F and G immune cells responded in a dose-dependent manner to homologous peptide but not to irrelevant peptides, thus confirming that F and G contain T cell epitopes of distinct specificity. We also analysed the proliferative responses of these T cell lines to the 38 kDa protein known to be present in MTSE and H37Ra organisms. Both preparations were able to induce proliferation of the G, but not of the F specific T cell line. This result corroborates the lack of proliferation to peptide F observed with LN cells from mice after immunisation with the recombinant 38 kDa protein.

DISCUSSION.

Despite containing the lipoylation site, peptide H failed to induce T cell proliferative responses and peptides A, B, D and E were also non-immunogenic, despite the presence of an amphipathic site within their sequence. Immunisation with the synthetic peptides revealed that peptides F and G were able to induce T cell proliferative responses in vitro and the results obtained with long-term T helper cell lines specific for peptides F and G provide additional evidence that these peptides contain T cell epitopes.

The influence of H-2 haplotype on peptide recognition was examined in different inbred strains of mice and peptides F and G were recognised in $H\text{-}2^b$, $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes (Table 1).

TABLE 1

Summary of proliferative responses to peptides C, F and G in different inbred mice

MOUSE STRAIN	PRIMING ANTIGEN	H-2 HAPLOTYPE	PROLIFERATIVE RESPONSES IN VITRO TO PEPTIDES		
			C	F	G
C57BL/10	GT-38	b	+[b]	-	++
C57BL/10	PEPTIDE[a]	b	-	++	++
B10A(3R)	PEPTIDE	b	ND	++	++
B10.BR	PEPTIDE	k	-	+++	++
B10.D2	PEPTIDE	d	ND	++	++
BALB/c	PEPTIDE	d	-	+	++

[a]Priming of mice and in vitro challenge with homologous peptide.

[b]Stimulation indices: (max. cpm with peptide/cpm without peptide)
+=<4;++=4-6;+++=>6.

Responses to peptides: A,B,D,E and H were negative in all strains.

These findings suggest that genetic promiscuity exists in T cell receptor recognition of peptide-MHC complexes. Similar results have been reported for synthetic peptides from other antigens [Brett, S.J. et al., Eur. J. Immunol., 19: 1303 (1989), Jardim, A. et al., J.Exp. Med., 172 : 645 (1990)] and may reflect intrinsic factors of T cell recognition sites [Margalit, H. et al., J. Immunol., 138: 2213 (1987)].

After immunisation with the recombinant CT38 fusion protein, proliferative responses were observed in vitro to peptides C and G, but not to peptide F.

A number of possible mechanisms might account for the failure of GT38-primed LN cells to proliferate in response to peptide F. These include, (i) the presence of proximal suppressor determinants, (ii) antigenic competition with other determinants, or (iii) structural differences between naturally processed antigen fragments and synthetic peptides [Brett, S.J. et al., J. Exp. Med., 168, 337 (1988), Ria, F. et al., Nature (London), 343: 381 (1990)]. Although peptide C was not stimulatory in vitro for LN cells primed with the same peptide, it was able to induce in vitro proliferative responses of LN cells from mice primed with the whole GT38 antigen. This may be due to the processing of the GT38 protein resulting in a peptide with additional flanking sequences which enhance the affinity of peptide-binding to MHC molecules. Alternatively, following immunisation with peptide, the epitope may be more susceptible to destruction by proteolytic enzymes.

The results obtained with LN cells from mice immunised with killed H37Ra and infection with BCG provide additional evidence that peptides C and G contain epitopes recognised by T cells. The response to peptides C and G following BCG infection indicates these epitopes are shared by M. tuberculosis and M. bovis. A T cell epitope is believed to be located in the M. tuberculosis 38 kDa antigen between residues 118 - 284 [Haslov, K. et al., Scand. J. Immunol., 31 : 503 (1990)]; this sequence overlaps with peptides B, C, D and E and the epitope may, therefore overlap with epitope C (201-220) described in this study. The effective presentation of peptide G, but not F, following processing of the 38 kDa antigen contained in MTSE and H37Ra was further supported by data from the peptide-specific T

cell lines.

In several experiments peptide G induced a variable proliferative response in vitro with LN cells from PBS/IFA immunised control mice. This effect was never observed with any of the other peptides. As proliferation was still observed with LN cells depleted of T cells with anti- Thy 1.2 mAb and complement, this proliferative response is tentatively attributed to B cells. This effect may be due to mitogenicity of the peptide or may reflect presensitisation of B cells by environmental exposure to the homologous PhoS protein constituent of E.coli.

Example 2

Elicitation of delayed type hypersensitivity (DTH) response in mice sensitised with mycobacterial and nonmycobacterial immunogens and challenged with peptide G

It is now widely accepted that the DTH methodology provides a good "in vivo" measurement of T-cell immunity. In the present study, it was planned to observe DTH response in mice sensitised with different mycobacterial and nonmycobacterial immunogens and challenged with peptide G.

Mice (6-8 weeks old) were pretreated with cyclophosphamide injected subcutaneously in the back at 50mg/kg body weight and 2 days later were injected subcutaneously with mycobacterial and nonmycobacterial immunogens. Either sonicated extracts or heat-killed or irradiated cells were used as immunogens. Each immunogen was emulsified in Incomplete Freunds Adjuvant (IFA) with a volume ratio of 1:1 and injected in a total volume of 200μl. The DTH response was elicited 2 weeks later with peptide G with a challenge dose of 5μg per mouse suspended in 20μl phosphate buffered saline (PBS) inoculated in the left hind pad of each animal from all immunogen groups and the PBS control group. Simultaneously, the same volume of PBS was injected in the right hind pad as a control.

The DTH response of mice was assessed by measuring foot pad swelling using a dial caliper (Pocotest, reverse spring loaded Caliper). The swellings were measured at 0,12,24,48,72,96 and

120 hours following challenge and the increase in foot pad thickness (mm^{-1}) between the challenged and nonchallenged foot pad of each mouse was recorded.

Initial foot pad swelling was observed at 24 hours in all groups of mice sensitised either with mycobacterial or nonmycobacterial immunogens and challenged with peptide G. In mycobacterial immunogen groups the peak swellings could be recorded at between 48 and 72 hours followed by gradual fall whereas the initial swellings reduced considerably up to 72 hours in nonmycobacterial immunogen groups. The results are shown in Table 2. The experiment was repeated, BALB/c and CDA/Ca mice being immunised as before and challenged either with Peptide G or PPD. The results are shown in Table 3.

TABLE 2

Mice immunised with:	Dose	Mean foot pad swelling (mm^{-1} ± Standard deviation at a time (hrs) after challenge with peptide					
		12	24	48	72	96	120
M. tuberculosis	10^6cells	0	2.7±0.3	4.9±0.8	5.1±1.0	3.3±0.8	1.7±0.6
BCG	10^6cells	0	3.0±0.7	4.8±0.6	5.1±0.2	3.9±0.4	1.1±0.4
M. avium	50μg	0	1.5±0.5	1.7±0.3	1.8±0.8	0.9±0.5	0
M. Scrofulaceum	50μg	0	2.8±0.8	2.1±0.4	1.9±0.5	0	0
M. vaccae	50μg	0	1.1±0.4	2.0±0.5	2.0±0.5	0	0
S. typhimurium	10^6cells	0	1.3±0.3	0.6±0.2	0.6±0.2	0	0
C. parvum	50μg	0	0.8±0.3	1.6±0.4	0.6±0.2	0	0
PBS control	-	0	0.2±0.2	0	0	0	0

TABLE 3

Immunogen	Time after Challenge (hrs)	Mean foot pad swelling (mm^{-1} ± standard deviation)		
		Peptide G for strain (5 mice/group)		PPD for both strains (10 mice/group)
		BALB/c	CBA/Ca	
PPD	24	3.6±0.5	3.6±0.9	3.3±0.7
	48	5.6±0.5	6.6±1.1	5.3±0.8
	72	5.0±1.0	5.6±1.1	5.7±0.7
	96	2.6±0.5	3.0±1.6	3.7±0.8
	120	1.2±0.4	1.6±0.9	
BCG	24	4.4±0.9	3.8±0.8	3.4±1.0
	48	7.4±0.9	6.8±1.1	6.5±1.0
	72	7.0±1.9	6.4±1.1	7.1±1.1
	96	4.6±1.7	4.2±0.8	6.2±0.8
	120	3.0±1.4	3.0±0.7	
M. tuberculosis	24	3.2±0.4	2.6±0.5	3.2±0.8
	48	8.0±0.7	7.2±0.8	5.7±0.5
	72	9.0±0.1	8.4±0.5	6.7±0.8
	96	7.8±0.8	7.4±1.5	6.1±1.3
	120	5.6±0.9	4.4±1.3	

Example 3

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll-Hypaque gradient centrifugation. The PBMC were suspended in culture medium (5% human A^+ serum, 2 mM glutamine and antibiotics in RPMI 1640 medium).

The concentrations of cells was adjusted and $2x10^5$ cells/well plated onto 96-well flat-bottom microtiter plates. Peptide G was added to a final concentration of 25 or 5 μg/ml (in triplicate). The final volume was 0.2 ml/well. After incubation for 6 days at 37°C, each well received 0.5 μ Ci ^3H Thymidine and was further incubated for 16 hrs. Cells were harvested onto glassfibre filters, washed and measured for radioactivity in a liquid scintillation counter. The whole procedure follows the general method described by Kaleb, B. et al., Eur J. Immunol., 20: 2651(1990).

The PPD reactivity of each donor was determined by standard skin testing with 10 U PPD/site (Evans PPD, injected i.d. in the forearm) and following the reaction daily for 3 days.

The results are shown graphically in Fig. 2, radioactive counts per minute (cpm) being plotted on a logarithmic scale for cells from PPD positive individuals (a,c) or PPD negative individuals (b,d) following in vitro culture in the presence of either 25μg/ml (a,b) or 5μg/ml (c,d) of peptide G.

Example 4

Proliferative responses to synthetic peptides derived from the 38 kDa antigen

Seventy-nine subjects were studied. PPD-positivity was established by skin induration diameters of more than 10 mm 48 h after inoculation with 10 U of PPD (Evans Med. Ltd, Langhurst, England). Ten subjects were healthy PPD-negative and 31 were healthy PPD-positive. Thirty-six patients with active tuberculosis were diagnosed by routine clinical, bacteriological

and histological parameters at the Lister Unit, Northwick Park Hospital. Blood was drawn either before or within two weeks after the onset of chemotherapy. Nineteen of these patients suffered from active pulmonary tuberculosis, 17 from extrapulmonary tuberculosis (the infection was localized in the lymphatic system in 9 patients, the other 8 patients suffered from tuberculous infection of the bone (2), breast (2), tuberculous pleuritis (3) and neck abscess (1)). None of the patients had evidence of HIV infection. Patients and healthy donors were of similar sex, race and age distribution but were not specifically matched.

Purified protein derivative (100,000 units/ml) was purchased from Evans Medical Ltd. (Langhurst, England), Concanavalin A from Sigma (St. Louis, MO, USA). A recombinant fusion protein expressing part of the 38 kDa protein (without the first 42 residues from the amino terminus and the last four from the carboxy terminus, thus not including sequences corresponding to peptides 38.A and 38.H, see below) and the carboxy-terminal-two-thirds of glutathione-S-transferase (GST-38) was prepared as described by Vordermeier et al, J. Immunol. (1991) 147, 1023. The insoluble GST-38 protein was partially purified by low speed centrifugation to pellet the fusion protein, followed by solubilization in 8 M urea and then dialysis against PBS. SDS-PAGE analysis indicated that about 60% of the total protein concentration of this preparation represented the fusion protein.

The following peptides were synthesized by simultaneous multiple BOC-synthesis.

38.A (1-20) MKIRLHTLLAVLTAAPLLLA, **38.H** (24-43) CGSKPPSGSPETGAGAGTVA, **38.I** (65-83) FNLWGPAFHERYPNVTITA, **38.E** (230-249) GNGGMVTGCAETPGCVAYIG, **38.K** (325-342) DAATAQTLQAFLHWAITDGN, **38.G** (350-369) DQVHFQPLPPAVVKLSDALI.

Truncated peptide derivatives of 38.G were prepared using standard methods known in the art. For the pepscan analysis described below, peptides were synthesized and supplied sterile in soluble form (0.2ml=~50μg/peptide) by Cambridge Research

Biochemicals (Northwick, England).

Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll Hypaque (Pharmacia, Uppsala, Sweden) from freshly drawn, citrated blood. The cells were suspended in RPMI 1640 supplemented with 2mM 1-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, and 5% A^+ heat-inactivated human serum. Two x 10^5 PBMC/well were cultured in the presence of the appropriate antigen in 96 well microtiter plates for 6 days at 37°C. Thirty-seven kBq of [^{3}H]-thymidine (Amersham International, Amersham, England) was added during the last 16 h of culture. Incorporated [^{3}H]-thymidine was quantitated by liquid scintillation counting. The antigen concentrations were 10 units/ml for PPD and 25 μg/ml in the case of GST-38. Synthetic peptides were used at 50, 25, 10 and 1 μg/ml. For pepscan analysis of peptide 38.G 10 μl/well of each peptide preparation was used. Quadruplicate determinations were performed for all antigens.

The mean proliferative responses of PBMC obtained from different groups of patients after stimulation with ConA and PPD are shown in Table 4. Functional viability of PBMC was confirmed by pronounced proliferative responses to the T cell mitogen Con A in all 77 subjects tested. Lymphocytes isolated from PPD skin test-negative healthy individuals did not respond to challenge with PPD in culture whereas all those derived from PPD-positive healthy individuals did. Responder status to PPD and subsequently to synthetic peptides was based on at least a 3-fold increase of cpm in cultures with antigen over cultures containing medium alone. Positive response to PPD were also obtained in 18 out of 19 patients with pulmonary tuberculosis, in 7 out of 8 patients with nonlymphatic extrapulmonary tuberculosis and in all patients with lymphatic tuberculosis. Although the mean cpm in patients with lymphatic tuberculosis was somewhat higher than the mean cpm for the two other groups this quantitative increase in proliferation was not significant. The proliferative responses to the recombinant fusion protein of the 38 kDa antigen (GST-38) were not demonstrable in PPD-negative healthy individuals, whereas the values in PPD-positive healthy individuals as well in

pulmonary and tuberculosis patients were significantly raised.

The proliferative response of PBMC to synthetic peptides tested at concentrations ranging from 1-50 μg/ml were found to be optimal at concentrations between 25 and 50 μg/ml. None of the 10 PPD-negative healthy individuals tested did respond to challenge with any of the peptides with the exception of one individual who responded weakly to peptides I and K. PPD-positive healthy subjects produced strong proliferative responses to peptide G in more than 90% of the individuals tested. In addition a substantial proportion of individuals responded to peptides A (86%), I (58%), E (44%), and K (61%), indicating the genetically permissive nature of their recognition.

A selective unresponsiveness to peptide G was observed in 89% of patients with pulmonary tuberculosis and in 75% of patients with nonlymphatic extrapulmonary tuberculosis. The selective nature of this anergy is evident by the fact that responsiveness to the other most immunogenic peptides A, I, E and K decreased only marginally or not at all in these patients. The mean proliferative responses shown in Table 4 further demonstrate this selective anergy to peptide G. The decrease of proliferative responses of pulmonary and nonlymphatic extrapulmonary tuberculosis patients to peptide G compared to healthy PPD-positive individuals was highly significant ($p<0.001$), whereas the mean proliferative responses of lymphatic tuberculosis patients did not differ from those obtained in healthy PPD-positive individuals. The difference in response to peptide G between extrapulmonary and lymphatic tuberculosis patients was also significant ($p<0.05$).

TABLE 4

MEAN PROLIFERATIVE RESPONSES OF PBMC ISOLATED FROM HEALTHY INDIVIDUALS AND TUBERCULOSIS PATIENTS[a].

PEPTIDE	PPD⁻HEALTHY	PPD⁺HEALTHY	PULMON. TB	EXTRAPULMON. TB (NONLYMPHATIC)	LYMPHATIC TB
A. Proliferative responses to PPD, ConA, and GST-38.					
PPD (10U/ml)	796[b] (1.68)	14195 (2.38)**[c]	13381 (2.71)**	12141 (5.75)**	21232 (2.63)**
ConA (5µg/ml)	22520 (1.23)	25650 (2.11)	17875 (2.50)	21938 (3.22)	18023 (1.69)
GST-38	276 (1.85)	2665 (2.71)**	1110 (2.58)*	922 (2.71)*	1635 (4.48)**
B. Proliferative responses to synthetic peptides from the 38 kDa antigen.					
38.A	383 (2.33)	2779 (2.41)**	2058 (2.43)**	2183 (3.2)**	2215 (2.09)**
38.I	671 (2.24)	1737 (2.50)*	2062 2.2)*	1310 (2.3)*	3361 (2.71)*
38.E	468 (2.01)	1422 (2.29)**	1199 (2.07)*	1225 (1.71)*	1549 (2.48)*
38.K	579 (1.87)	2212 (2.58)*	1691 (2.38)*	2406 (3.04)*	2295 (1.9)**
38.G	445 (2.24)	4279 (3.00)**	765 (1.90)	805 (2.8)	2837 (2.40)**
Medium	365 (1.23)	445 (1.08)	464 (1.09)	419 (1.18)	407 (1.22)

a) PBMC were cultured for 6 days at 2 x 10^5/well in quadruplicates in the presence of 50 µg/ml peptide or 25 µg/ml GST-38 for 6 days. [^{3}H]-thymidine incorporation was measured during the last day of culture.

b) Geometric mean of [^{3}H]-thymidine incorporation expressed in cpm with x + SD in brackets.

c) Difference compared to healthy PPD-negative group: $p < 0.05$ (*) or $p < 0.001$ (**).

Example 5

Determination of the epitopic core-region of peptide 38.G

In view of the permissive recognition as well the decreased response in patients with pulmonary tuberculosis it was of interest to determine the structure of the core-epitope within peptide G. For this purpose a pepscan 'window analysis' was performed using a series of 25 overlapping 15mer peptides covering the sequence of residues 336-374. Proliferative responses of PBMC isolated from two healthy PPD-positive responders showed that the core regions comprising 12 residues 351-362 and 11 residues 352-362, respectively, differed in length only by a single residue (Q) at the N-terminus. Having established the approximate position of the core structure within the sequence of the original 20mer of peptide G we set out to determine the minimal structure capable to induce proliferation of PBMC from different 38.G responders. Truncated versions of peptide G ranging in size from 8-16 amino acid residues and covering the 20mer were synthesized. PBMC from 16 healthy PPD-positive responders to peptide G, including the two already assayed by pepscan analysis, were incubated with these peptides. Lymphocytes from all 16 individuals responded to the synthetic peptide of 10 amino acid residues length (residue 353-362) but not to the peptide of 8 residues (354-361). Peptides 12-16 residues long also induced lymphocyte proliferation of similar magnitude.

Example 6.

PBL were obtained on a voluntary basis from laboratory donors. Laboratory donors represented a diverse range of ethnic backgrounds, some of whom originated from tuberculosis endemic countries. All such donors were apparently healthy and had no previous history of clinical tuberculosis. A total of 20 laboratory donors (14 males and 6 females), ranging in age from 25 to 57 were included in the study. Patient blood specimens were obtained with consent from individuals attending an infectious diseases clinic at Northwick Park Hospital, London. Blood samples were only obtained from patients who were confirmed or strongly suspected of having tuberculosis. Diagnosis of

tuberculosis was made on the basis of both clinical examination and standard bacteriological tests. A comprehensive clinical history comprising results of chest X-rays, sputum smears, mantoux tests and BCG scars was provided for each patient. Patients were classified on clinical grounds as suffering pulmonary or extrapulmonary (lymphatic) tuberculosis. A total of 34 patients were included in the study, most of whom were of Asian or African descent. Males and females were equally represented and patient ages ranged from 19 to 74.

Peptides were obtained as described above in Example 4. Proliferative response to the peptides were also measured by the methods described in Example 4.

(i) Proliferative responses in healthy humans and tuberculosis patients to 19.6A and 19.7.

Four subject groups were chosen for analysis of T cell proliferative responses (i) PPD healthy individuals (ii) PPD^+ healthy individuals (iii) pulmonary tuberculosis patients and (iv) lymphatic tuberculosis patients (Fig. 3). T cells from PPD^- donors failed to respond when cultured in vitro with either peptide or with PPD. However, pronounced responses to Con A and CWE were demonstrable (data not shown). Uniformly strong T cell proliferative responses to p19.6A were observed in all PPD^+ individuals. These responses to p19.6A were significantly increased ($p<0.001$) when compared to the PPD^- group. Although quantitative differences were observed among PPD^+, pulmonary and lymphatic groups they were not significant. It should be noted that the response to p19.6A was in many instances equivalent in magnitude to that of PPD. Given the 100% responder status in PPD^+ individuals (Table 5), it is implicit that p19.6A is able t associate with multiple different class II HLA molecules. In contrast, responses to p19.7 in PPD^+ healthy subjects and pulmonary patients were considerably weaker (Fig 3) although significantly elevated in about 50% of subjects when compared to the PPD^- group. Interestingly, the response to p19.7 was significantly higher ($p<0.005$) and more frequent (90%) in lymphatic tuberculosis patients (Table 5).

TABLE 5

Table II. Human T cell proliferative responses to peptides 19.6A and 19.7.

Status	p19.6A			p19.7		
	cpm[a]	Total[b]	% responders[c]	cpm	Total	% responders
PPD$^-$	1157	8	0	792	12	0
PPD$^+$	18093	15	100	2108	20	50
Pulmonary TB	10394	9	100	2000	15	53
Lymphatic TB	29798	6	100	6622	10	90

[a]Geometric mean of thymidine incorporation. [b]Total number of individuals in each group tested. [c]% positive responders (cpm + peptide > mean cpm without peptide + 3 x SD).

(ii) Epitope cores recognized by human and murine T cells.

Given that p19.6A and p19.7 overlap by four amino acid residues (VTGS) we analysed two important questions regarding epitope specificity; (i) do p19.6A and p19.7 possess a shared or distinct determinant (ii) do responder T cells from man and mice recognize the same or different core epitopes within p19.6A and p19.7. To address these questions, we tested a series of overlapping synthetic peptides (PEPSCAN) which spanned the sequence of p19.6A and P19.7. LNC from B10.BR mice (obtained from Olac Harlem (Shows Farm, Bicester, Oxon, UK) primed with either p19.6 or p19.7 were cultured in vitro with the overlapping series of peptides. LNC from p19.6A primed mice responded in vitro specifically to peptides spanning 47-61 to 54-68 and all intervening peptides. hence, the core P19.6A epitope spans residues 54-61. Similar analysis with p19.7 immune LNC revealed a core epitope spanning residues 71-78. These data clearly demonstrate that in the context of the H-2^k haplotype, p19.6A and p19.7 contain distinct epitope cores which are completely non overlapping. To determine whether the same or different core epitopes were recognized by human T cells, PBL from a healthy donor were cultured with the same series of peptides. The results illustrated that human T cells recognize essentially the same core epitopes identified in the mouse. The results from these experiments, showing the localization of the two epitopes within the amino acid sequence are summarized in Figure 4.

(iii) DTH skin reaction in humans.

Given that p19.6A and p19.7 induced strong proliferative activity in vitro, we reasoned they might also induce a DTH reaction in humans. In a pilot experiment, two PPD^+ and one PPD^- laboratory volunteers were injected intradermally in the forearms with PPD, p19.6A and p19.7 and diameters of induration and erythema measured at 24, 48 and 72 h. The PPD^- subject did not develop a DTH reaction in response to either peptide or PPD. In contrast, PPD and p19.6A produced marked induration (>10mm) and erythema in both PPD^+ subjects which peaked at 48h and then subsided. Peptide 19.7 failed to induce a reaction in either of the two subjects tested.

CLAIMS

1. A peptide comprising the sequence (I'):

   His-Phe-Gln-Pro-Leu-Pro-Pro-Ala-Val-Val, (I')

   or a variant or immunological equivalent thereof.

2. A peptide comprising the sequence (I)

   Asp-Gln-Val-His-Phe-Gln-Pro-Leu-Pro-Pro- (I)
   -Ala-Val-Val-Lys-Leu-Ser-Asp-Ala-Leu-Ile

   or a variant or immunological equivalent thereof.

3. A pharmaceutical or veterinary composition comprising a solution or suspension, in a suitable solvent, of a peptide according to claim 1 or 2 or a variant or immunological equivalent thereof.

4. A peptide according to claim 1 or 2 or a variant or immunological equivalent thereof or a composition according to claim 3 for use in a method of diagnosis practiced on the human or animal body.

5. The use of a peptide according to claim 1 or 2 or a variant or immunological equivalent thereof or a composition according to claim 3 in the preparation of a medicament for use in a method of diagnosis practiced on the human or animal body.

6. A process for producing a compositions comprising the peptide according to claim 1 or 2 or a variant or immunological equivalent thereof, which comprises admixing the peptide with a suitable solvent, diluent or carrier therefor.

7. A method of diagnosing tuberculosis in a human or non-human animal suspected of suffering from tuberculosis, said method comprising either:

(a) intradermal injection of an effective, non-toxic amount of a peptide according to claim 1 or 2 or a variant or immunological equivalent thereof or a composition according to claim 3 to said human or non-human animal; or

(b) contacting lymphocytes from the human or non-human animal with a lymphocyte-activating amount of a peptide according to claim 1 or 2 or a variant or immunological equivalent thereof or a composition according to claim 3.

8. A diagnostic kit comprising a peptide according to claim 1 or 2 or a variant or immunological equivalent thereof or a composition according to claim 3.

9. A method of distinguishing a TB patient from an infected or otherwise sensitised but healthy clinical suspect which comprises:

(i) obtaining a sample of the patients lymphocytes;

(ii) performing a lymphocyte activation test using as antigen a peptide according to claim 1 or 2;

(iii) performing a second lymphocyte activation test using as antigen a second peptide from the 38 kDa antigen of M. tuberculosis; and

(iv) comparing the results of the first and second tests to establish whether the test of step (ii) above is anergenic.

10. A method according to claim 9 wherein the second peptide comprises the sequence (II):

Phe-Asn-Leu-Trp-Gly-Pro-Ala-Phe-His-Glu-
Arg-Tyr-Pro-Asn-Val-Thr-Ile-Thr-Ala (II)

or a variant or immunological equivalent thereof; or comprises the sequence (III):

Asp-Ala-Ala-Thr-Ala-Gln-Thr-Leu-Gln-Ala-Phe-
Leu-His-Trp-Ala-Ile-Thr-Asp-Gly-Asn (III)

or a variant or immunological equivalent thereof; or comprises the sequence (IV):

Met-Lys-Ile-Arg-Leu-His-Thr-Leu-Leu-Ala-Val-
Leu-Trp-Ala-Ala-Pro-Leu-Leu-Leu-Ala (IV).

11. A peptide comprising the sequence (II), (III) or (IV) as defined in claim 10 or a variant or immunological equivalent thereof.

12. A pharmaceutical or veterinary composition comprising a solution or suspension, in a suitable solvent, of a peptide according to claim 11 or a variant or immunological equivalent thereof.

13. A peptide according to claim 11 or a variant or immunological equivalent thereof or a composition according to claim 12 for use in a method of diagnosis practiced on the human or animal body.

14. The use of a peptide according to claim 11 or a variant or immunological equivalent thereof or a composition according to claim 12 in the preparation of a medicament for use in a method of diagnosis practiced on the human or animal body.

15. A process for producing a compositions comprising a peptide according to claim 11 or a variant or immunological equivalent thereof, which comprises admixing the peptide with a suitable solvent, diluent or carrier therefor.

16. A method of diagnosing tuberculosis in a human or non-human animal suspected of suffering from tuberculosis, said method comprising either:

(a) either intradermal injection of an effective, non-toxic amount of a peptide according to claim 11 or a variant or immunological equivalent thereof or a composition according to claim 12 to said human or non-human animal; or

(b) contacting lymphocytes from the human or non-human animal with a lymphocyte-activating amount of a peptide according to claim 11 or a variant or immunological equivalent thereof or a composition according to claim 12.

17. A diagnostic kit comprising a peptide according to claim 11 or a variant or immunological equivalent thereof or a composition according to claim 12.

18. A monoclonal antibody against a peptide as defined in any one of claims 1, 2 or 11.

19. A peptide comprising the sequence 19.6A:

Gly-Ala-Ala-Ser-Gly-Pro-Lys-Val-Val-Ile-
Asp-Gly-Lys-Asp-Gln-Asn-Val-Thr-Gly-Ser (19.6A)

or a variant or immunological equivalent thereof.

20. A pharmaceutical or veterinary composition comprising a solution or suspension, in a suitable solvent, of a peptide according to claim 19 or a variant or immunological equivalent thereof, or a peptide comprising the sequence 19.7:

Val-Thr-Gly-Ser-Val-Val-Cys-Thr-Thr-Ala-
Ala-Gly-Asn-Val-Asn-Ile-Ala-Ile-Gly-Gly (19.7)

or a variant or immounological equivalent thereof.

21. A peptide according to claim 19 or a variant or immunological equivalent thereof, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof, or a composition according to claim 20 for use in a method of diagnosis practiced on the human or animal body.

22. The use of a peptide according to claim 19 or a variant or immunological equivalent thereof, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof, or a composition according to claim 20 in the preparation of a medicament for use in a method of diagnosis practiced on the human or animal body.

23. A process for producing a compositions comprising a peptide according to claim 19 or a variant or immunological equivalent thereof or as defined in claim 20 or a variant or immunological equivalent thereof, which comprises admixing the peptide with a suitable solvent, diluent or carrier therefor.

24. A method of diagnosing tuberculosis in a human or non-human animal suspected of suffering from tuberculosis, said method comprising either:

(a) intradermal injection of an effective, non-toxic amount of a peptide according to claim 19 or a variant or immunological equivalent thereof, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof, or a composition according to claim 20 to said human or non-human animal; or

(b) contacting lymphocytes from the human or non-human animal with a lymphocyte-activating amount of a peptide according to claim 19, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof, or a variant or immunological equivalent thereof or a composition according to claim 20.

25. A diagnostic kit comprising a peptide according to claim 19

or a variant or immunological equivalent thereof, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof, or a composition according to claim 20.

26. A monoclonal antibody against a peptide as defined in any one of claims 19 or 20.

27. A method of distinguishing a TB patient from an infected or otherwise sensitised but healthy clinical suspect which comprises:

(i) obtaining a sample of the patients lymphocytes;

(ii) performing a lymphocyte activation test using as antigen a peptide according to claim 1 or 2;

(iii) performing a second lymphocyte activation test using as antigen a second peptide, which peptide is as defined in claim 19 or a variant or immunological equivalent thereof, or a peptide as defined in claim 20 or a variant or immunological equivalent thereof; and

(iv) comparing the results of the first and second tests to establish whether the test of step (ii) above is anergenic.


1
MKIRLHTLLAVLTAAPLLLAAAGCGSKPPSGSPETGAGAGTVATTPASSPVTLAETGST
59
A (1-20)
H (24-43)
60
LLYPLFNLWGPAFHERYPNYTITAQGTGSGAGIAQAAAGTVNIGASDAYLSEGDMAAHK
118
119
LGMNIALAISAQQVNYNLPGVSEHLKLNGKVLAAMYQGTIKTWDDPQIAALNPGVNLPG
177
B (146-165)
178
TAVVPLHRSDGSGDTFLFTQYLSKQDPEGWGKSPGFGTTVDFPAVPGALGENGNGGMV
235
C (201-220)
E
D (210-229)
236
TGCAETPGCVAYIGISFLDQASQRGLGEAQLGNSSGNFLLPDAQSIQAAAAGFASKTPA
294
E (230-249)
F (285-304)
295
NQAISMIDGPAPDGYPIINYEYAIVNNRQKDAATAQTLQAFLHWAITDGNKASFLDQVH
353
F
G
354
FQPLPPAVVKLSDALIATISS
374
G (350-369)

Fig.1.

Fig. 2.

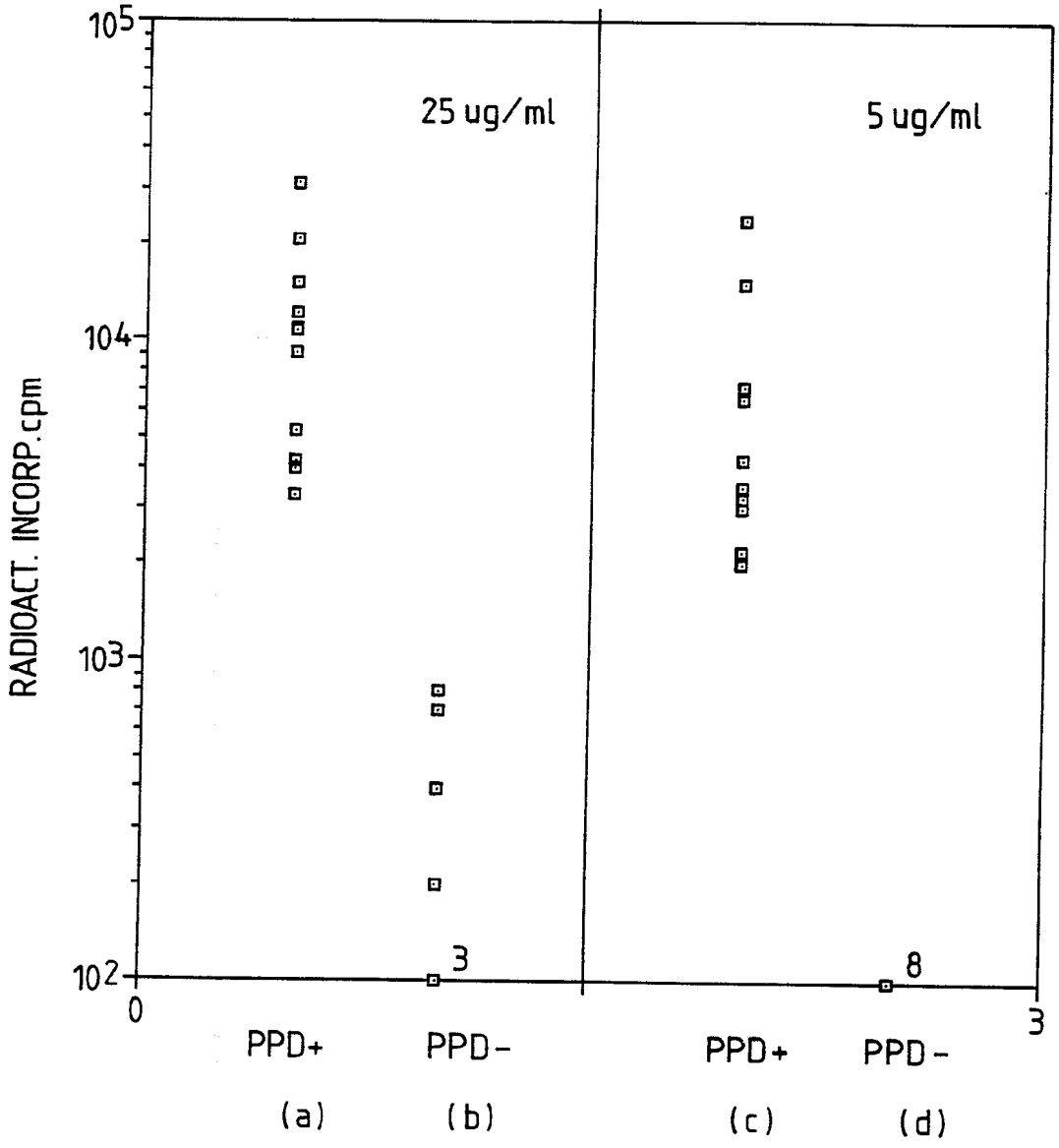

10^5
10^4
10^3
10^2
RADIOACT. INCORP. cpm
25 ug/ml
5 ug/ml
3
8
0
3
PPD+
PPD-
PPD+
PPD-
(a)
(b)
(c)
(d)

Fig. 3.

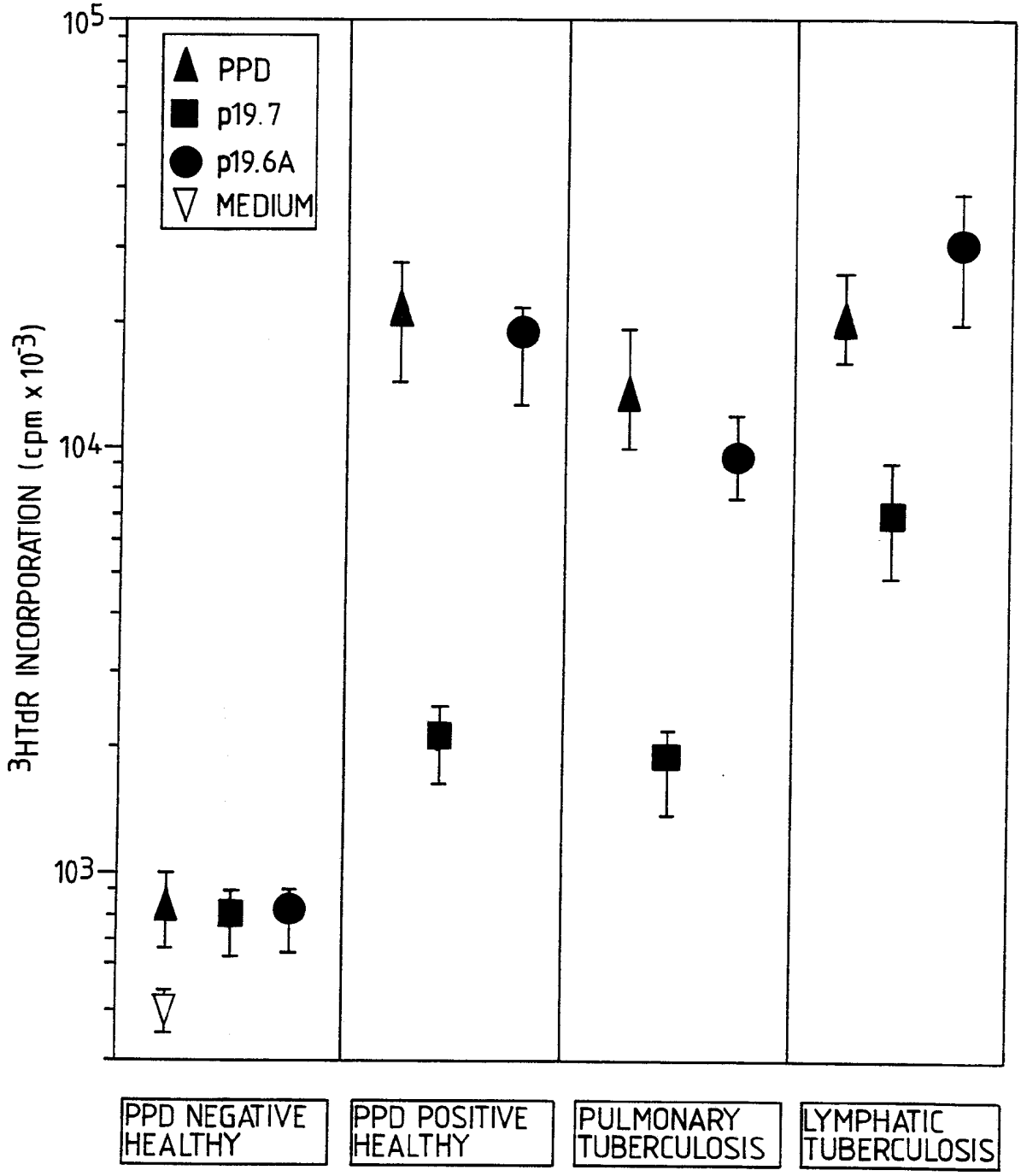
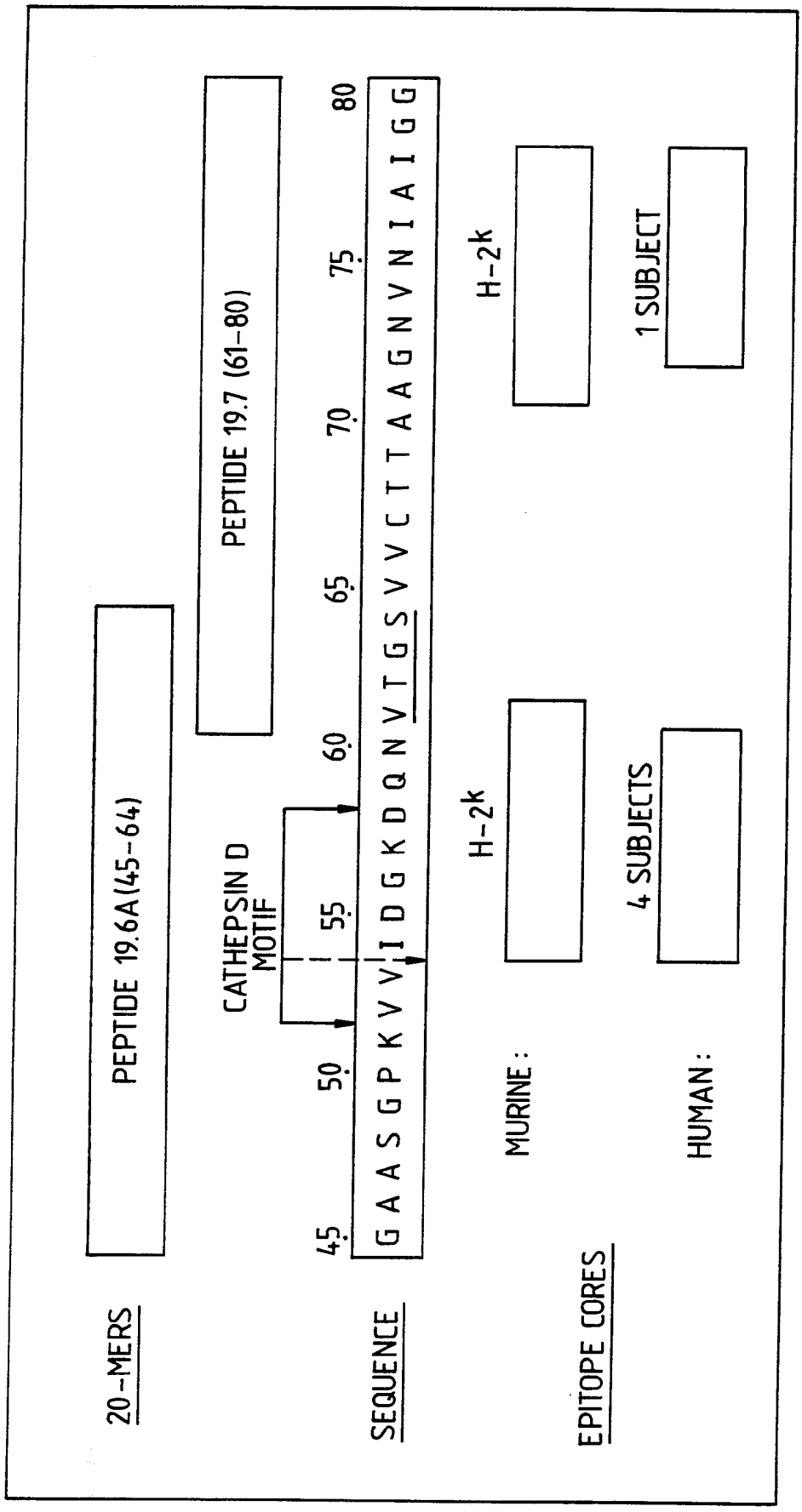

PPD
p19.7
p19.6A
MEDIUM
10^5
10^4
10^3
^{3}HTdR INCORPORATION (cpm x 10^{-3})
PPD NEGATIVE HEALTHY
PPD POSITIVE HEALTHY
PULMONARY TUBERCULOSIS
LYMPHATIC TUBERCULOSIS

Fig.4.


20-MERS
PEPTIDE 19.6A(45-64)
PEPTIDE 19.7 (61-80)
SEQUENCE
45
50
55
60
65
70
75
80
G A A S G P K V V I D G K D Q N V T G S V V C T T A A G N V N I A I G G
CATHEPSIN D MOTIF
EPITOPE CORES
MURINE:
H-2k
HUMAN:
4 SUBJECTS
H-2k
1 SUBJECT